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Chirality in Microcystins by Thermospray Ionization

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ABSTRACT

The chiralities of the amino acids present in the blue-green algal peptides (microcystins) were determined by the hydrolysis of the peptides, followed by derivatization with Marfey's reagent and separation of the diastereomeric derivatives over a reverse-phase HPLC column and detection by Thermospray-MS techniques.

INTRODUCTION

Most of the naturally occurring peptides and proteins are made up of l-amino acids. However, some of the toxic peptides have also been known to contain d-amino acids.[1] Blue-green algal (Cyanobacteria) toxic peptides (Microcystins) were found to comprise two variant l-amino acids along with few invariant d-amino acids, based on their nmr data and GC separation over a chiral-Val capillary column.[1] During our investigations on toxic peptides originating from various species of cyanobacteria, we required more efficient and sensitive procedure to establish the chiralities of the amino acids present in these unknown peptides.

As a result of our investigations we have developed a procedure involving the LC/MS technique. The diastereomeric derivatives of the hydrolysates, with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's agent),[2] were chromatographed over a commonly used reverse phase C_{18} column. The eluents were subjected to thermospray ionization and analyzed either under total or selected ion monitoring modes. L-phenylalanine was used as the internal standard for quantification of the amino acids present in various hydrolysates of the blue-green algal peptides. The observed sensitivity for most amino acids was in the picomole range. The unknown peptides were found to have two variant l-amino acids (L,R or R,R) and three d-amino acids (A,D,E or A,B,M-asp) which corroborated with results observed in other blue-green algal peptides.[3]

EXPERIMENTAL

The entire investigation was carried out using Finnigan-MAT TSQ 4600 tandem mass spectrometer, Waters 600 (for separations) multi-solvent delivery system, Waters 510 (for post column

additions) solvent delivery system, and Vestec Thermospray ionization source.

The reagents and solvents used were either analytical or HPLC grade. The HPLC grade solvents were purchased from Burdick and Jackson, Muskegon, MI. The hydrolysis tubes and Marfey's reagent were purchased from Pierce, Rockford, IL. Amino acid standards were purchased from Sigma Chemical company, St. Louis, MO.

Hydrolysis of Peptides: Peptide solution (1-5 nMole) was placed in the hydrolysis tube and freeze dried. The residue was treated with 6N HCl (0.5-1.0 ml) and degassed several times and filled with nitrogen and sealed. The solution was kept in a heating block maintained at 150°C for 24 hours. The solution was cooled and freeze dried. The residue was kept in the freezer at -20°C until use.

Derivatization with Marfey's Reagent: Amino acid standards or hydrolysates of peptides (200 pmole - 50 nmole) in aqueous solution (50-100 μl) was treated with 1% solution of Marfey's reagent in acetone (60% excess than the required amount) followed by 1.0 molar aqueous bicarbonate solution (10-50 μl). The reaction mixture was heated at 40°C in a heating block for 1 hour, cooled, and treated with 2M HCl (20 μl). The pH of the resulting solution was adjusted to be 2 or less with additional 2M HCl if required. The solution was degassed and diluted with adequate amounts of water prior to LC/MS analysis.

LC/MS Analysis of Diastereomeric Derivatives: Standard or hydrolysate derivative solution (50 μl) was injected into a C_{18} column and eluted (1.5-2 ml/min.) with 0.1% ammonium acetate (A, pH 6) and acetonitrile (B) with a linear gradient of 0-50% B in 20 minutes. The eluents were sprayed through the thermospray interface and ionized. Optimum temperature for the source block and the probe tip heater were determined to be 269°C and 271°C, respectively. In order to maintain the aerosol temperature at 250-235°C through the analysis, the probe tip was kept at 260-248°C manually. The ions were analyzed either under selected ion monitoring mode or scanning from m/z 300 to 500 every second.

RESULTS AND DISCUSSIONS

Seven membered toxic cyclic peptides have been isolated from fresh water blue-green algae (cyanobacterium).[1,3,4] All of them were found to contain five commonly occurring amino acids along with two unusual amino acids, ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) and methyldehydroalanine (Mdha).[1,4] In all the known seven membered blue-green algal peptides three invariant amino acids, d-alanine, erythro-ß-methyl-d-isoaspartic acid (Masp), are present.[1,3,4] The two variant amino acids (Xaa, Yaa) are present in l-configuration in various combinations such as Leu & Arg or Tyr & Arg or Arg & Met or Arg & Ala. The general structure of these peptides may be assigned as

cyclo-D-Ala-L-Xaa-D-Masp-L-Yaa-Adda-D-Glu. The stereochemistry of Adda in microcystims was assigned to be 2S, 3S, 8S, 9S based on the chemical cleavage and synthesis of Adda.[5] We had earlier reported certain blue-green algal peptides in which variant amino acids were found to be Arg & Arg.[3] The two earlier termed as invariant residues ß-Masp and Mdha were replaced by Asp and Dha, respectively.[3] Hence, we required sensitive and reliable methodology to assign the chirality of most of the amino acid residues, including the new amino acids detected for the first time, present in the microcystins.

Enantiomers have identical physical and chemical properties including retention properties over a chromatographic column. However, diastereomers of the compound with two asymmetric center could be resolved over a chromatographic column. Hence, enantiomers of an amino acids with one asymmetric center could be distinguished by introducing another asymmetric environment in the molecules either by derivatizing with an optically active reagent or inducing intermolecular interaction with a HPLC chiral stationary phase.[2,6] The commercially available HPLC chiral column were found to be unsuited for our purpose involving the detection by thermospray-MS methods. Hence, we have selected the former approach and introduced the additional chiral center in the amino acid components of the blue-green algal peptides by derivatizing with 1-fluoro-2,4-dinitrophenyl-5L-alanine amide (Marfey's or FDAA reagent).

Individual D- and L- isomer standards for all except ß-Masp were commercially available. Commercial ß-Masp standard was a mixture of D- and L- isomers of erythro and threo forms. Initially, derivatives of D- and L- isomers of alanine, arginine, aspartic acid, glutamic acid, leucine, ß-methylaspartic acid, with Marfey's reagent were prepared to record the mass spectra of the enantiomers. The derivatives originating from L- isomers shall elute prior to the ones from their corresponding D-isomers. The derivatives were chromatographed over reverse phase C_{18} column. The eluted components were subjected to on-line thermospray ionization and their corresponding mass spectra were recorded scanning from m/z 300-550. Optimum pH of the ammonium acetate buffer was determined to be 6 from the observed ion counts by maintaining the buffer pH at various levels.

The molecular ions of the FDAA derivatives (Figure 1,2) indicated them to be formed by the reaction of the fluoro group of the reagent with the amino group of the amino acid and subsequent elimination of hydrofluoric acid.[2] Hence, the cross linkage should be at position 1 of the phenyl ring of the reagent. The mass spectra of the derivatives indicated the molecular ion adduct with the ammonia is more pronounced, with the exception of arginine derivative, than the corresponding molecular ion. The adducts with acetic acid, sodium and pottasium were also observed. In the case of leucine, methanol adduct was also detected.

Other ions in the mass spectra indicate them to be fragments

originating either from molecular ion or its ammonium adduct. The fragmentation led to the loss of 17 (NH_3), 18 (H_2O), 30 (HCHO), 32 (CH_3OH), and 46 (*HCOOH or* C_2H_5OH) *from both.* In the case of arginine derivative, the observed loss of 60 and 85 is assigned due to the loss of groups at the side chain. In the case of alanine, only the loss of water was detected which might well be due to the scanning of limited m/z range (>300). In the isomers of glutamic acid and ß-methylaspartic acid, a peak at m/z 336, which might be originating by the loss of 18 and 32 from the molecular ion or by the loss of 17,18 and 32 from the ammonium adduct, was observed. However, the relative abundance of m/z 336 was different between the isomers of Glu and ß-Masp. The mass spectra of the diastereomeric derivatives originating from the enantiomers seem to be similar with slight variance in the relative abundance of the ions. The ions selected for monitoring the amino acid derivatives, based on their relative abundances, are listed in Table 1.

The retention times of diastereomers of Glu and ß-Masp, with the same molecular weights, were determined separately under carefully controlled LC and selected ion monitoring MS conditions (m/z 417). ß-Masp has two asymmetric centers leading to four diastereomeric derivatives and Glu with one asymmetric carbon leading to two. Commercial ß-Masp was a mixture containing major amounts of threo D&L and minor amounts of erythro D&L diastereomers. The observed retention times were assigned to the four diastereomers accordingly.

Mixture containing all six diastereomers were analyzed under identical conditions. The six diastereomers were well resolved and could be identified unambiguously. Identical retention times were obtained on repetition of the analysis. In order to improve the sensitivity of detection, a multiple ion detection (MID) experiment was created in order to monitor the individual ions, by data system control, listed in Table 1 sequentially. Individual ions were monitored at equal intervals. The solution containing all the derivatives (50 μl) was separated over the C_{18} column and analyzed under the above LC and MID conditions. All derivatives (2.85 nmole) except less prominent diastereomers of ß-Masp, could be identified based on the observed ion chromatograms (Figure 3). Mixtures containing various amounts of the derivatives were analyzed under identical conditions in order to determine the detection limits. The observed values are also listed in Table 1. The relative retention times for the standards, with respect to that of D-Ala, listed in Table 2 were reproducible.

Blue-green algal peptides originating from Microcystis aeruginosa (collected at various geographical locations), Red oscillatoria and Green oscillatoria were hydrolysed with 6N HCl under nitrogen at 150°C for 24 hours and lyophilized. A reagent blank was also processed under identical conditions. The residue was derivatized with FDAA reagent. Standard derivatives followed by a reagent blank were analyzed under the above LC/MS conditions. The sample derivatives were also analyzed under identical conditions. The reagent blanks were analyzed in between each

sample run to check any contamination or carry over from earlier analysis. In every instance, the blank runs were free from any impurities. At the end of the last sample analysis, the standard analysis was repeated to examine any possible variance in the retention times. The results are listed in Table 2. Blue-green algal hydrolysate derivatives were also analyzed scanning from m/z 300 to 600 in order to confirm the observations from the full scan mass spectra. The identity of the amino acid in the sample was confirmed by comparison of the mass spectrum with the standard spectrum (Figure 4) and its chirality from the relative retention time of its FDAA derivative. These peptides isolated from Microcystis and Oscillatoria blue-green algae also contain the same sequences and chiralities of the amino acids as reported earlier in other microcystins.[1,3,4] All contain two variant amino acids in the L- form, two invariant amino acids in the D- form and either aspartic acid or its ß-methyl derivative in the D- or erythro D-form, respectively (Figures 5-8).

During the full scan analysis of the derivatized microcystins hydrolysates, spectra with base peak at m/z 552 were recorded (Figure 9). This could be accounted for the ion formed by the elimination of methanol from the quasi-molecular ion of ADDA. Neither the presence of the ammonium adduct or the protonated molecular ion of the ADDA-FDAA adduct were observed in the recorded spectra.

We had earlier reported the sequence of the toxic peptide isolated from Green Oscillatoria algae to be cyclo-D-Ala-D-Arg-D-isoAsp-L-Arg-ADDA-dehydroalanine.[3] One of the peptides isolated from red oscillatoria (MW, 1023) have been found to be cyclo-D-Ala-D-Arg-erythro-D-ß-Masp-L-arg-ADDA-dehydroalanine, based on similar approach reported earlier[3], and results observed during this investigation.

Experiments were also conducted in order to determine the chiralities and the ratios of the amino acids present in the blue-green algal peptides quantitatively. L-phenylalanine was selected as the internal standard based on its reported retention time[6], cost in comparison with its corresponding D- isomer, and most importantly its absence in any of the peptides investigated thus far. It was derivatized with FDAA reagent and the derivative was ionized under thermospray conditions and mass analyzed. The recorded full scan spectrum (m/z 300-550) indicated the fragmentation pattern to be similar to that observed for other amino acids. Ions due to the loss of NH_3, acetic acid (60), $C_6H_5^+$, (77) from quasi molecular ion and its corresponding ammonium adduct were observed. The observed sensitivity for the ammonium adduct was greater compared to the protonated molecular ion and that observed while same submolar quantities of other amino acid derivatives were analyzed. Hence, during quantification, a lesser abundant MH^+ ion was selected as the reference peak.

Standard solutions containing amounts ranging from 1.25 to 37.5 nmoles of the analytes and 5 nmoles of internal standard were

analyzed. Relative amounts of individual amino acid derivatives along with relative abundances were fitted into a linear regression curve. The determined regression constants, along with relative retention times with respect to L-phenylalanine derivative, for individual standard is listed in Table 3. Similarly, microcystin samples were also analyzed with L-Phe-FDAA derivative. Each sample run was preceded by a blank analysis as before. Observed relative abundances of detected amino acids were extrapolated into their corresponding calibration curves in order to obtain their amounts present in samples. The results are shown in Table 4. A linear relationship between the analyzed amounts and observed ion counts was observed during the analysis of standard derivatives. Hence, the discrepancy in the observed ratios may only be explained due to the error introduced during hydrolysis and the effect of heat on the released amino acids. It was previously reported that in addition to microcystin-LR, its corresponding demethylated (D-isoAsp) analogs was also isolated from Microcystis species found in Scotland, Canada and USA.[3] The formation of the latter was found to be temperature dependent. The observed smaller amount of D-Asp in one of the two 7820 (Scotland) peptides is due to the presence of the demethylated peptide.

CONCLUSIONS

Introduction of an additional asymmetric center in the amino acids by derivatizing with FDAA reagent is effective, reproducible, and quantitative. The diastereomeric derivatives are easily separable over a reverse phase column. Thermospray ionization and mass spectral analysis are useful in assigning the chirality of the amino acids in the blue-green algal peptides. Quantification may be possible under improved hydrolytic conditions and carefully planned standard addition procedures.

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Table 1. FDAA Derivatives.

AMINO ACID	MH^+ (m/z)	$(M+NH_4)^+$ (m/z)	MONITORED ION (m/z)	DETECTION LIMITS
D-Ala	342	359	359	400 pmole
L-Ala	342	359	359	400 pmole
D-Arg	428	444	427	20 pmole
L-Arg	428	444	427	20 pmole
D-Asp	386	403	403	400 pmole
L-Asp	386	403	403	50 pmole
D-Glu	400	417	417	400 pmole
L-Glu	400	417	417	200 pmole
D-Leu	384	401	401	3.0 nmole
L-Leu	384	401	401	1.5 nmole
ß-Masp*	400	417	417	-

* Detection limits unavailable since the diastereomeric standards are unavailable.

Table 2. Chirality

SAMPLE	LA	D-A	L-L	D-L	L-D	D-D	L-E	D-E	L-R	D-R	ßmD*
D&L Isomers	0.87	12.35	1.11	1.27	0.59	0.65	-	0.72	0.89	0.94	0.65
Green Osc.[1]	-	12.36	-	-	-	0.66	-	0.72	0.88	-	-
Red Osc.[2]	-	12.35	-	-	-	0.66	-	0.72	0.89	-	-
Red Osc.**	-	12.35	-	-	-	0.65	-	0.72	0.89	-	-
S23G1[3]	-	12.35	1.11	-	-	0.66	-	0.72	0.88	-	-
7820[4]	-	12.37	1.12	-	-	0.65	-	0.73	0.88	-	0.65
Akerstox[5]	-	12.36	1.11	-	-	-	-	0.73	0.88	-	-
Monroe[6]	-	12.36	1.11	-	-	0.67	-	0.73	0.88	-	0.65
S23G1**	-	12.37	1.10	-	-	0.67	-	0.73	0.88	-	0.66
D&L Isomers	0.87	12.37	1.11	1.26	0.60	0.66	-	0.73	0.88	0.94	0.65

*, ß-methylaspartic acid (erythro-D).
**, Crude sample

[1] Green Oscillatoria, Norway; [2], Red Oscillatoria, Norway; [3], Microcystis aeruginosa, Canada; [4], Microcystis aeruginosa, Scotland; [5], Microcystis aeruginosa, Norway; [6], Microcystis aeruginosa, Monroe, Wisconsin, USA.

From [3,4,6,] microcystin-LR and its corresponding demethyl analog peptide have been isolated.

Table 3. Linear Regression Analysis

Amino Acid Derivative	Ion (m/z)	RRT*	Correlation Coefficient	Intercept	Slope
L-Phe	418	1.00	-	-	-
L-Ala	342	0.80	0.9982	-0.03	0.4055
D-Ala	342	0.89	0.9986	0.11	0.7669
L-Arg	427	0.80	0.9927	-0.19	2.5770
L-Asp	386	0.55	0.9925	-0.13	0.4110
D-Asp	386	0.57	0.9917	-0.23	0.5734
L-Glu	400	0.69	0.9990	0.00	0.2976
D-Glu	400	0.74	0.9978	0.07	0.2650
L-Leu	384	0.98	0.9956	-0.16	0.7702
D-Leu	384	1.10	0.9915	-0.11	0.4756
ß-Masp, Threo, L-	403	0.62	-	-	-
ß-Masp, Erythro,L-	403	0.68	-	-	-
ß-Masp, Threo, D-	403	0.71	-	-	-
ß-Masp, Erythro,D-	403	0.78	-	-	-

*, Retention time of L-phe is 17.39 minutes.

Table 4. Measured Ratios.*

Sample	D-Ala	L-Arg	D-Asp	ß-Masp*	D-Glu	L-Leu
7820	1.00 (0.89)	0.07 (0.80)	-	3.3 (0.78)	1.33 (0.74)	1.01 (0.98)
7820 (Dup)	1.00 (0.89)	0.07 (0.80)	0.02 (0.68)	3.26 (0.78)	1.32 (0.74)	1.02 (0.98)
Akerstox	1.00 (0.89)	0.092 (0.80)	-	0.05 (0.77)	0.19 (0.74)	1.60 (0.97)

*, measured relative retention time with respect to L-phenylalanine (17.39 min.) are listed in parenthesis.

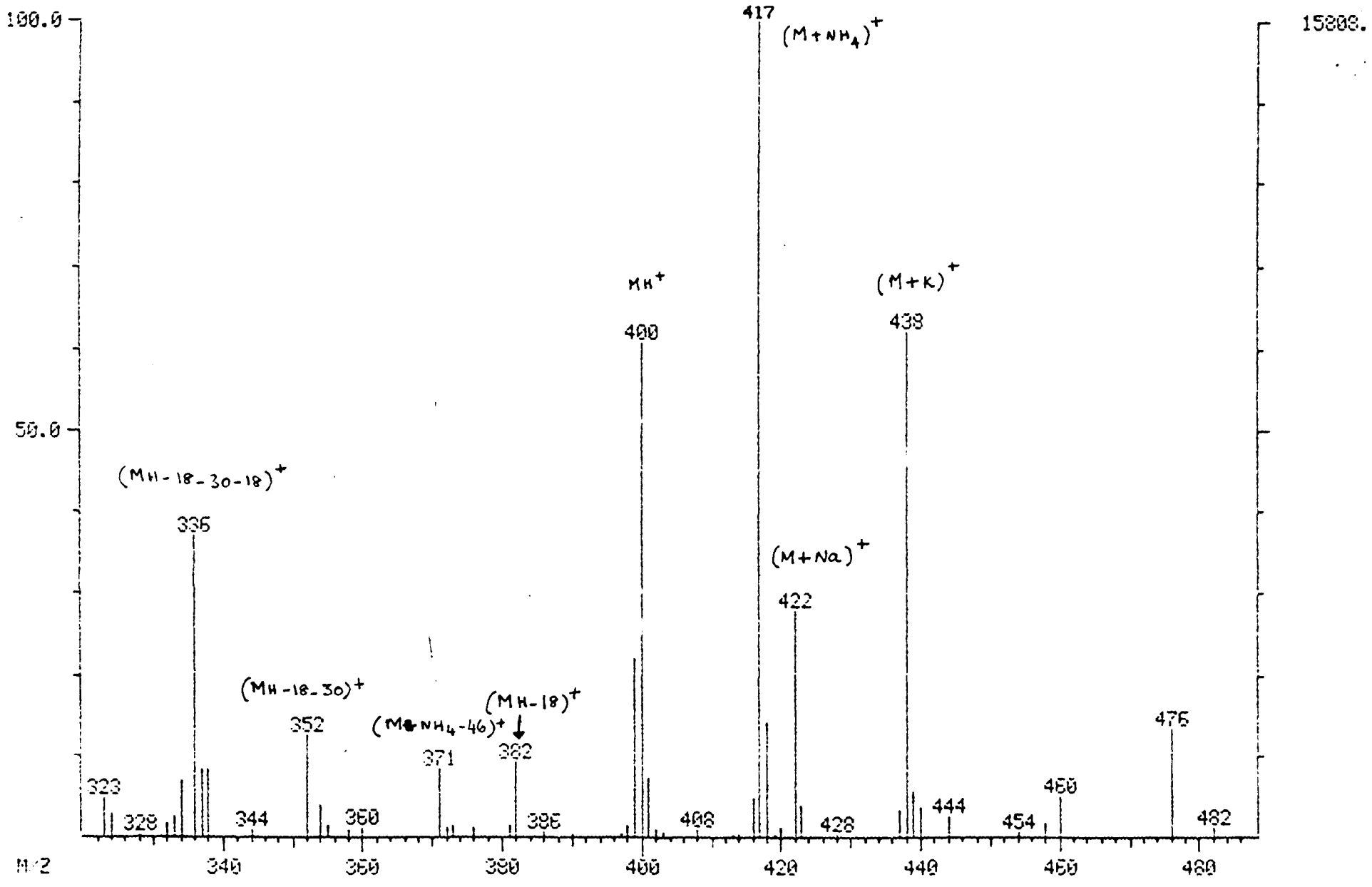

Figure 1. Thermospray Ionization Mass Spectrum of D-Glutamic Acid.

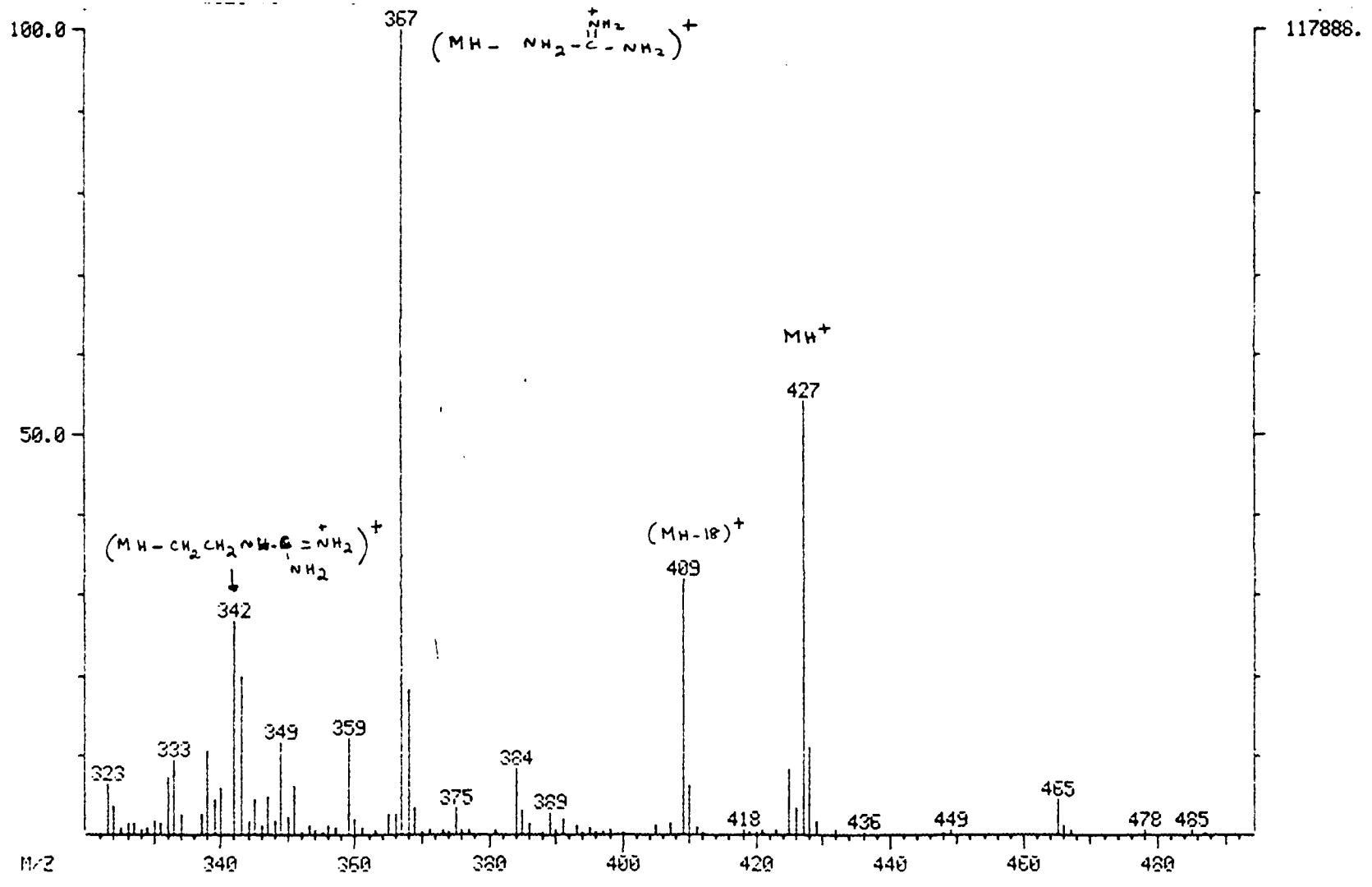

Figure 2. Thermospray Ionization Mass Spectum of L-Arginine.
(Thermospray conditions as in Figure 1)

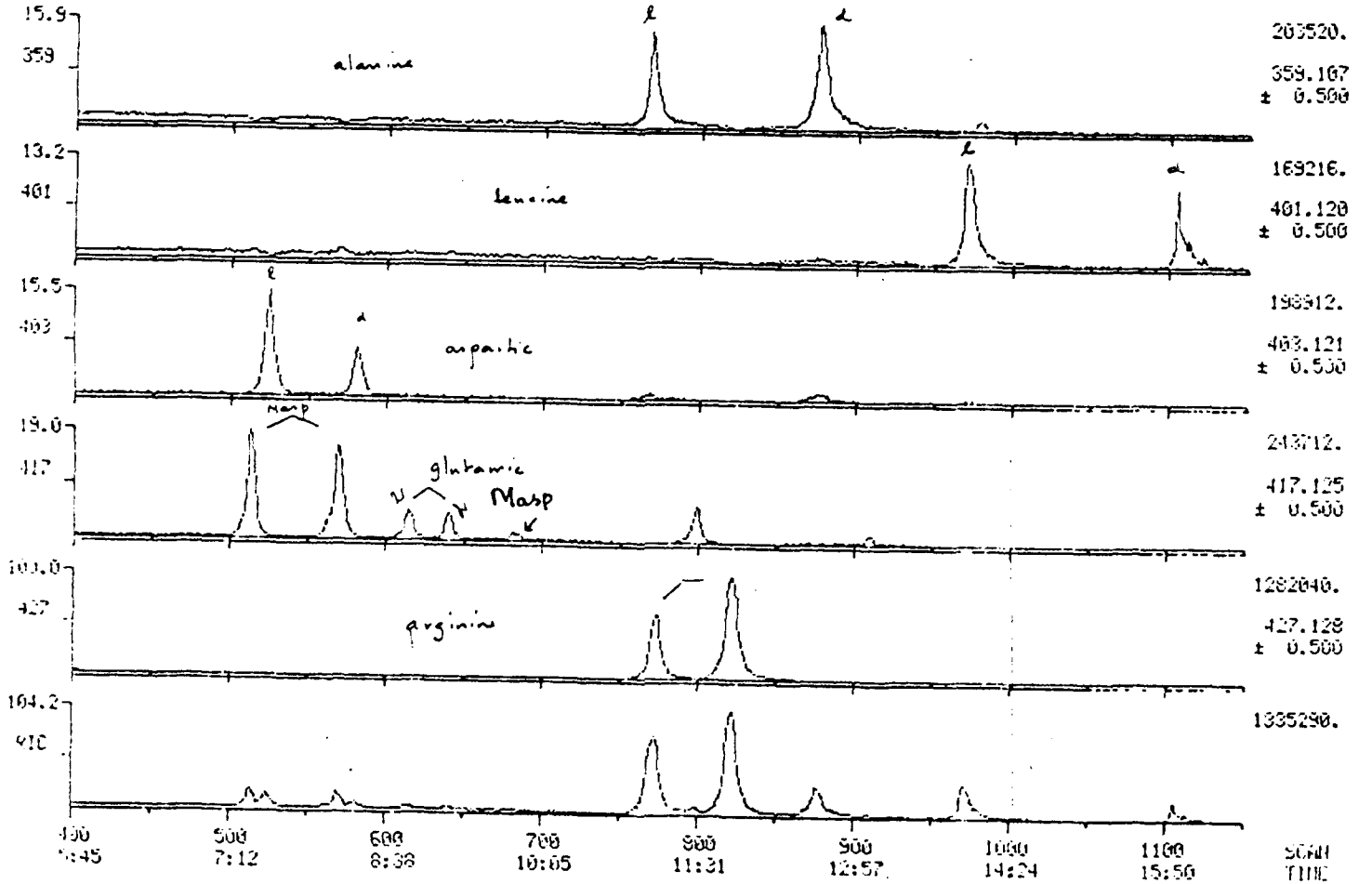

Figure 3. Mass Chromatograms of D&L Isomers
(C_{18} HPLC column (3.9mm, 25cm, 7μ), Solvent A, 0.1M NH_4OAC; Solvent B, Acetonitrile; 10-50% B in 5 minutes, 1.5 ml/min)

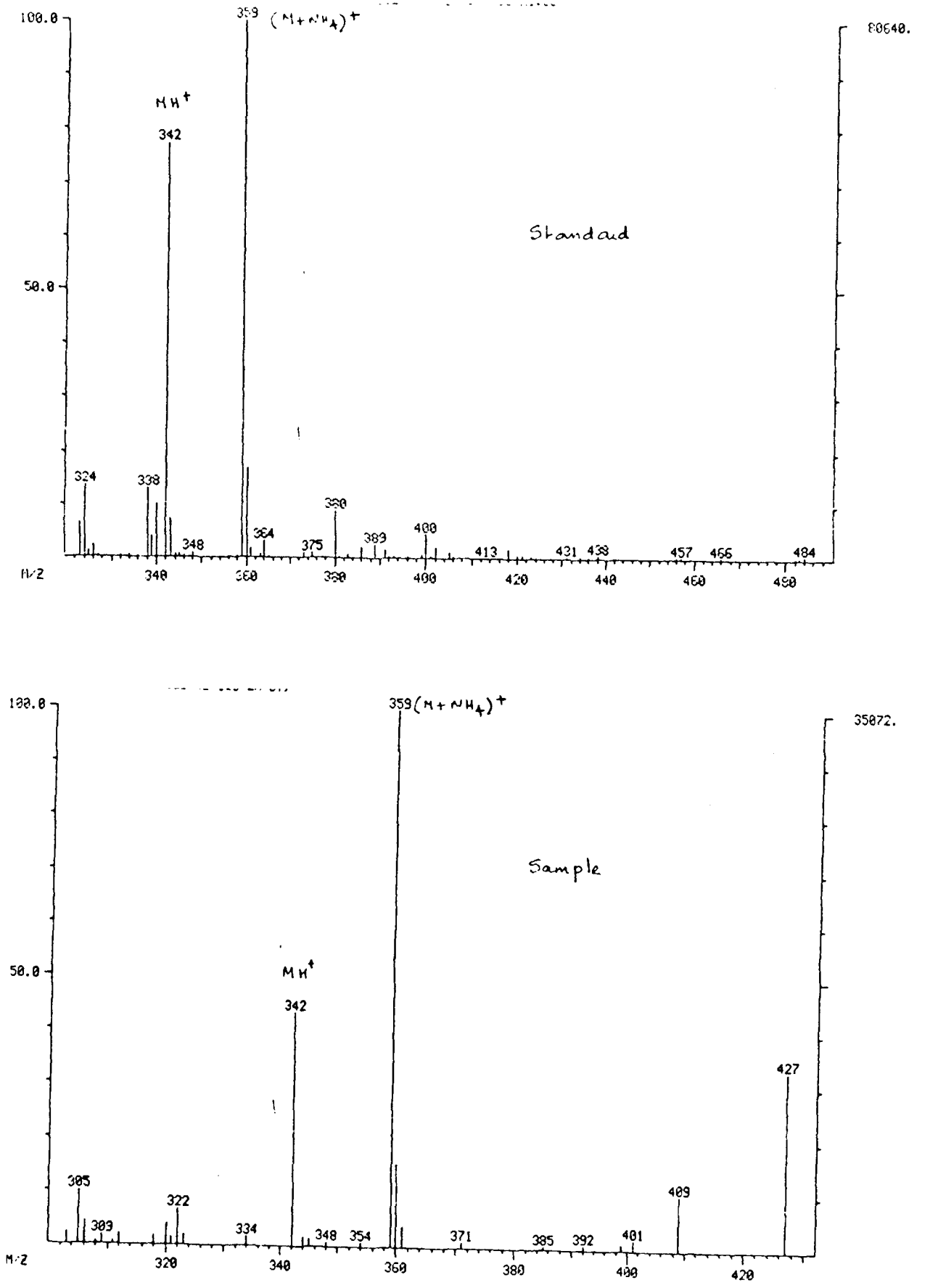

Figure 4. Standard and Sample (Green Oscillatoria) Spectra of D-Alanine-FDAA Derivativ

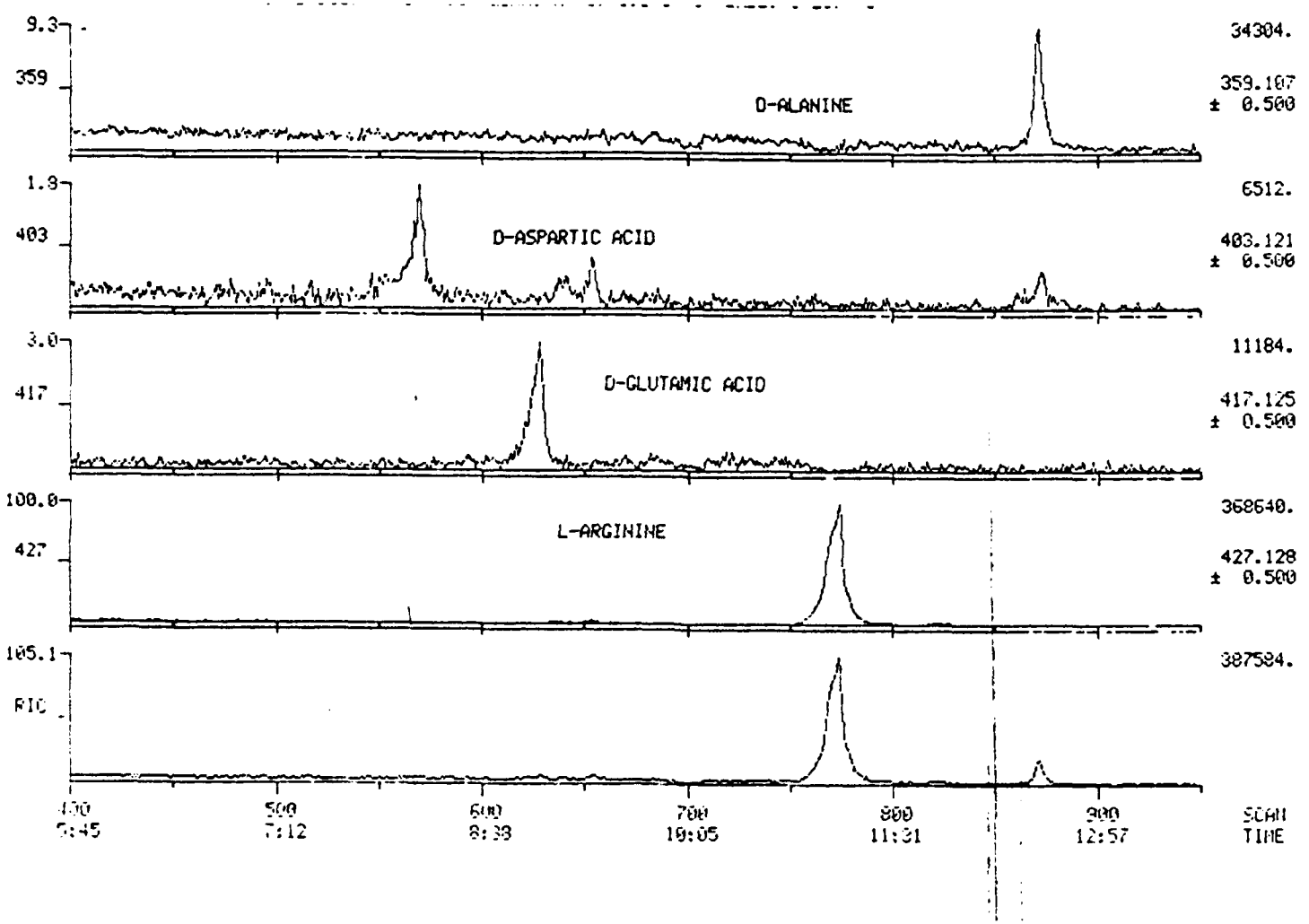

Figure 5. Mass Chromatogram of Red Oscillatoria Hydrolysate-FDAA Derivatives

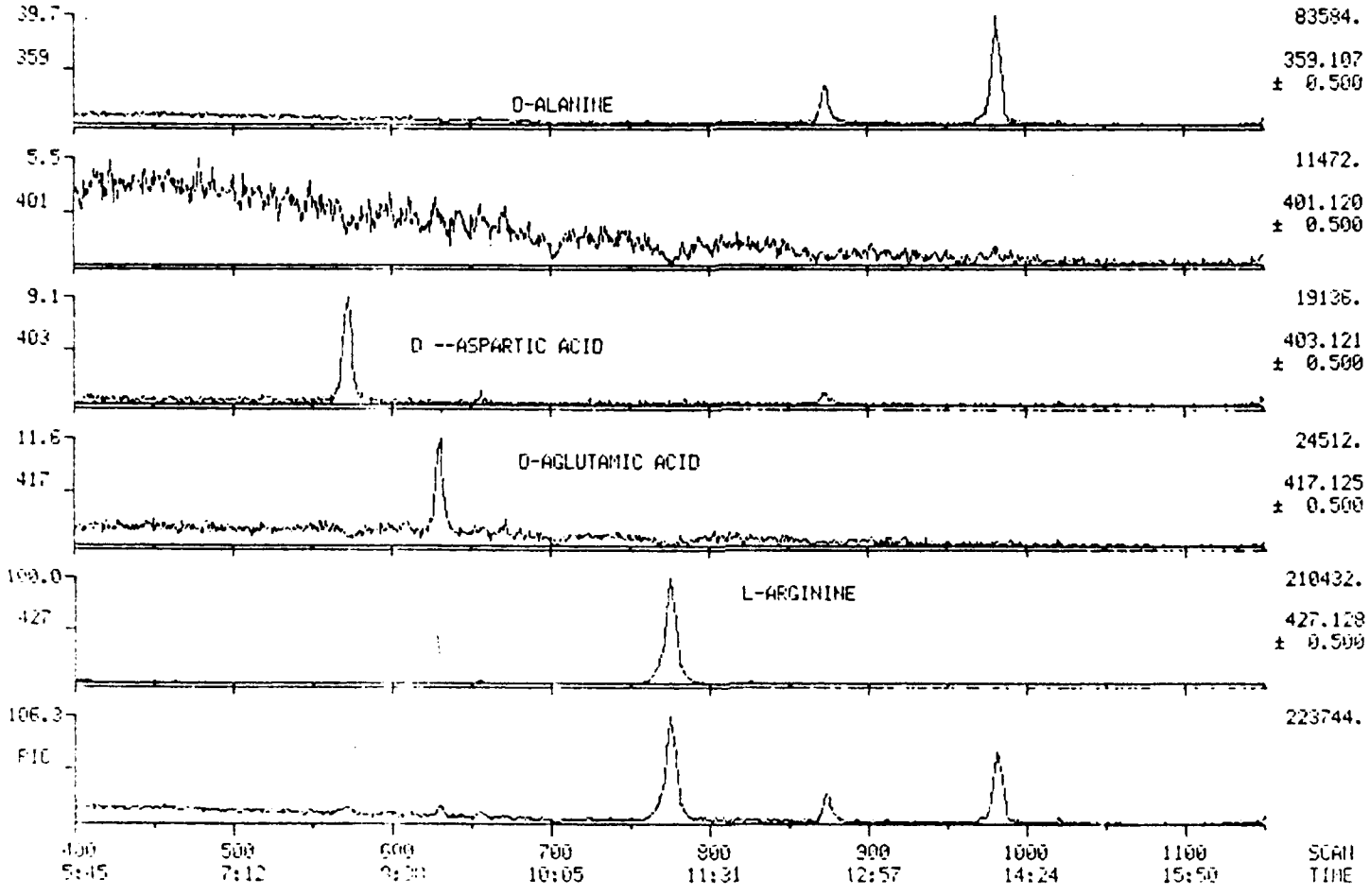

Figure 6. Mass Chroamtogram of Green Oscillatoria Hydrolysate-FDAA Derivatives.

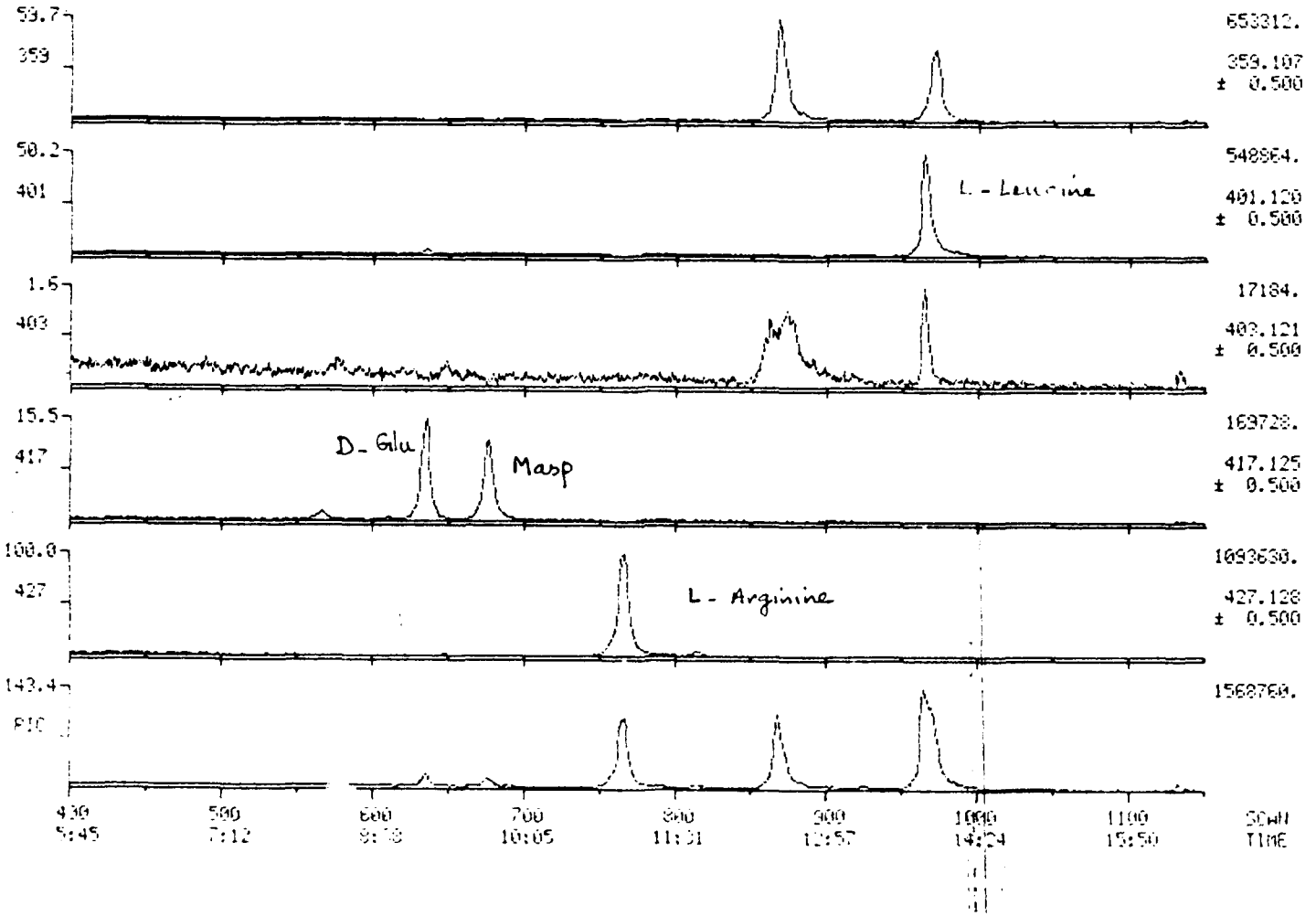

Figure 7. Microcystis aeruginosa (Monroe, Wisconsin) Hydrolysate-FDAA Derivatives

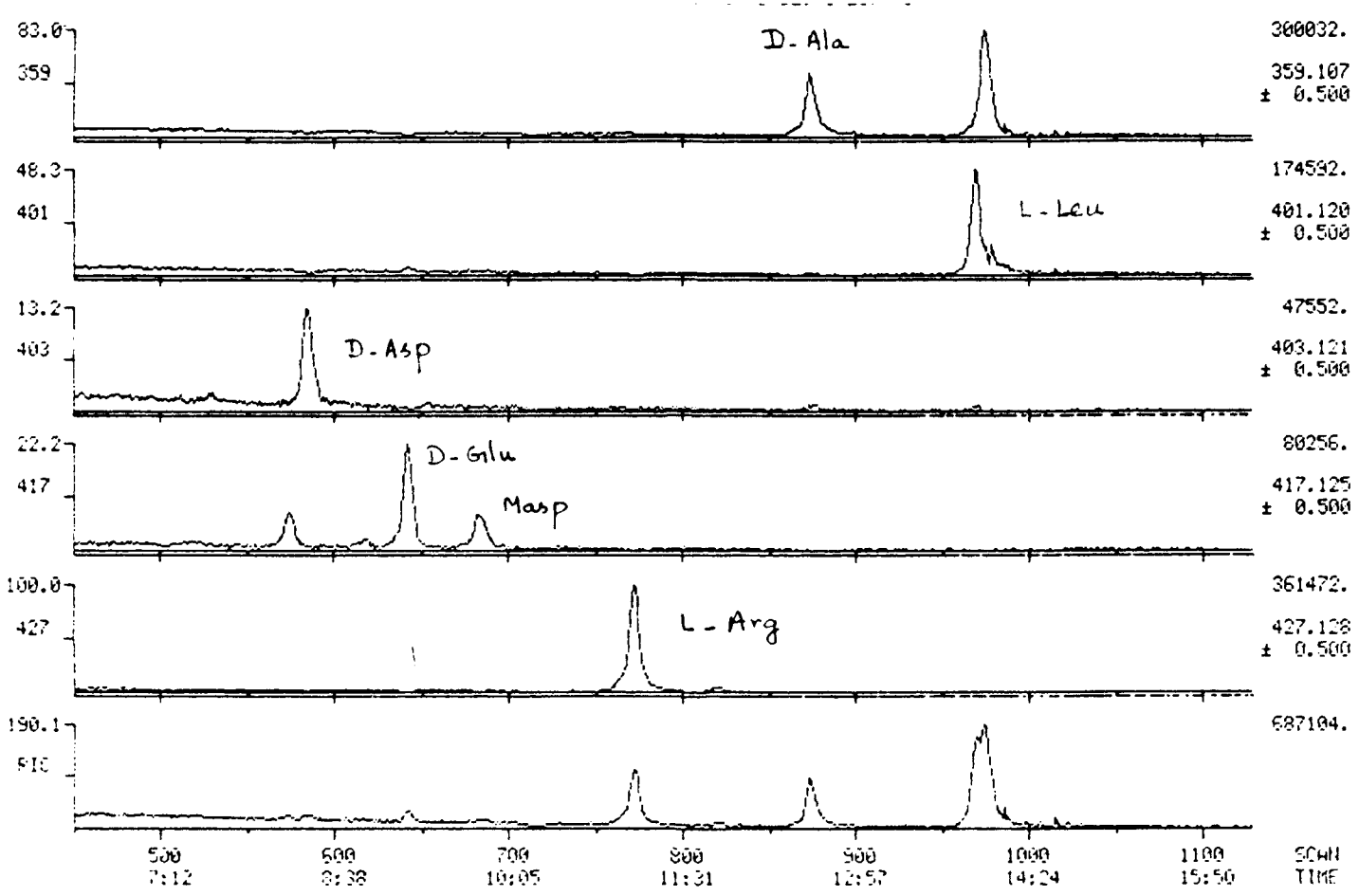

Figure 8. Microcystis aeruginosa (Canada) Hydrolysate-FDAA Derivatives.

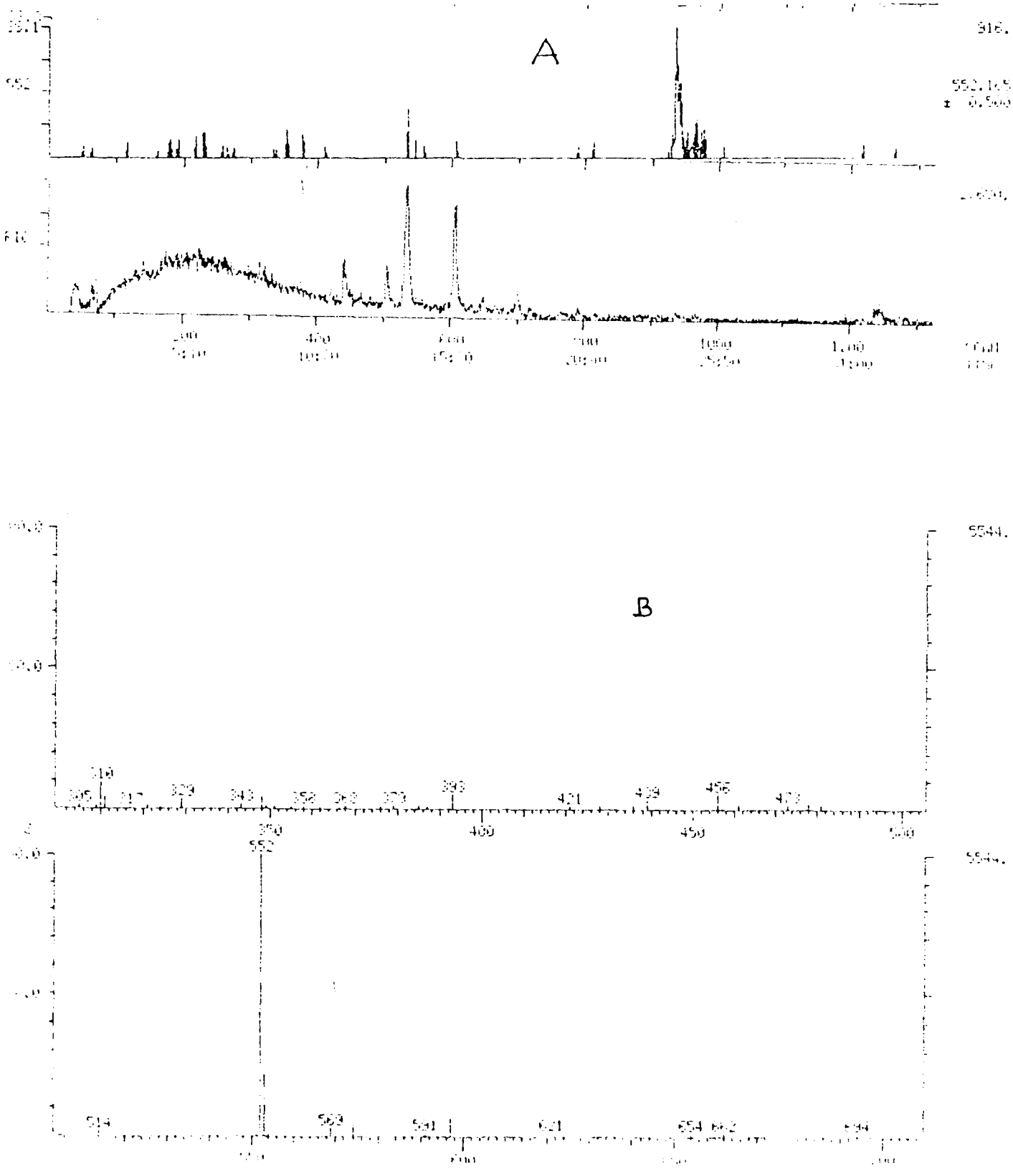

Figure 9. ADDA-FDAA Derivative
(A, Mass Chromatogram, MH+ -32; B, Mass Spectrum)